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SATURATION TRANSFER DIFFERENCE NMR AS AN ANALYTICAL TOOL FOR DETECTION AND DIFFERENTIATION OF PLASTIC EXPLOSIVES ON THE BASIS OF MINOR PLASTICIZER COMPOSITION

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14. ABSTRACT:

A novel, analytical saturation transfer difference (STD) NMR methodology has been developed to support the unambiguous identification and discrimination of Semtex and C-4 plastic explosive mixtures in complex matrixes on the basis of plasticizer composition. The approach is based on the ability of the STD NMR to completely eliminate all interfering NMR signals. Virtually extracting the proton spectrum of the plasticizers only (using their characteristic binding to serum albumin protein) enables the unambiguous structural identification. The results demonstrated that the observed STD chemical shifts of the plasticizers can be used as a reliable diagnostic target for the presence of the corresponding explosive mixture in complex matrices. The viability of the method was illustrated using control samples of commercially available plasticizers found in Semtex and C-4 explosive mixtures, either alone (pure) or in the presence of soil. Our novel approach was then successfully applied for the analysis of unknown explosive samples that had been sent to our laboratory for full characterization and explosive identification.

15. SUBJECT TERMS

Bovine serum albumin protein (BSA)	Detection	Plastic explosive mixtures
Saturation transfer difference (STD)	Differentiation	Specific binding
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PREFACE

The work described in this report was conducted under the Army Technology Objective R.FP.2010.01. It was started in March 2010 and completed in December 2011.

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SATURATION TRANSFER DIFFERENCE NMR AS AN ANALYTICAL TOOL FOR DETECTION AND DIFFERENTIATION OF PLASTIC EXPLOSIVES ON THE BASIS OF MINOR PLASTICIZER COMPOSITION

1. INTRODUCTION

The use of explosives by terrorists and criminals remains a serious concern. There is presently an urgent need for the development of improved methods for detection, identification, and chemical attribution of explosives. To date, many analytical methods have been developed for detecting bulk and trace explosives. Most of these methods are based on the detection of energetic components of explosive devices, such as 2,4,6-trinitrotoluene (TNT); 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX); pentaerythritol tetranitrate (PETN); 1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane (HMX); ethylene glycol dinitrate (EGDN); ammonium nitrate (AN); and nitrocellulose (NC).[1–4] Alternatively, in one recent study,[5] fluorescence-based techniques and statistical analyses were used to target the minor plasticizer composition of classic military and commercial explosives and thereby enable the detection and differentiation of Semtex and C-4 plastic explosive mixtures. This method was not direct, and the differentiation relied on the addition of fluorescent indicators and additives, which modified the environment of the plasticizers. The study demonstrated successful differentiation of the plasticizers in reference samples (pure Semtex and C-4 plastic explosive mixtures); however, when soil was present in the samples, the characteristic patterns used for explosive detection were shifted as compared with those for the reference materials. The authors[5] could not predict the nature of the eventual shifts that might be induced by different potential contaminates. Accurate identification of minor plasticizer composition will not only aid in the identification of plastic explosive mixtures; it could also provide important information for assigning attribution.[5,6]

The objective of our study was to use saturation transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy to provide a novel methodology for the unambiguous detection and identification of plasticizer composition of Semtex and C-4 plastic explosive mixtures. In contrast with methods currently in use,[5] our method is direct, does not require addition of fluorescent indicators or additives, and provides unambiguous identification of the plasticizers in authentic samples (which may exhibit strong background interference due to the presence of soil or other contaminants). The novelty of our study is the application of STD NMR as a spectral editing technique. STD NMR enables us to obtain simplified proton spectra that are based on the specific binding of the small plasticizer molecules (ligands) to bovine serum albumin (BSA) protein receptors. The implementation is straightforward: Once a small amount (in the picomol range) of BSA protein receptor is added to an unknown explosive sample, the STD NMR yields only signals for the bound plasticizers. All interfering proton signals from non-binding components of the explosive mixture and impurities present in the solution are effectively eliminated.

STD NMR spectroscopy[7–9] exhibits excellent sensitivity in discriminating between binders and non-binders. The method has been used extensively for ligand screening in drug design and discovery studies, for determining ligand-binding epitopes at atomic resolution, and for characterizing events of molecular recognition. Practically, the ligand STD signals can be

detected in a difference spectrum obtained by subtracting a spectrum with on-resonance protein saturation from a reference spectrum. The reference spectrum is collected with off-resonance irradiation that is outside of the proton spectral window, which results in no protein saturation. The difference spectrum results from the decrease in the ligand signal intensities (upon saturation of the protein) relative to those of the reference spectrum, due to transfer of saturation from the protein to the bound ligand. The saturation is transferred via an $^{1}H–^{1}H$ cross-relaxation pathway, also called spin diffusion, in which effectiveness is distance-dependent. The ligand protons that are bound to the receptor receive the highest degree of magnetization from the saturated receptor and as a result exhibit strong STD effects. Protons that are either 5 Å away from the receptor surface or do not bind at all reveal no STD NMR signals. This allows identification of binders or delineation of binding epitopes (areas of the ligand that actually contact the binding site).

Although STD experiments are widely used to identify binders or ligand-binding epitopes, in this study, we instead use the established binding of plasticizers to BSA protein to demonstrate that the resultant STD chemical shifts can yield unambiguous structural identification of the plasticizers present in unknown complex matrices, including the proton NMR signals of other components of the plastic explosive mixtures and any interfering background proton NMR signals from soil or other impurities. We demonstrate that the STD NMR method enables unambiguous plasticizer detection on an atomic level and plasticizer discrimination that is based on unique chemical structure and characteristic binding to BSA protein. The results of this study will enable investigators to discriminate between Semtex and C-4 plastic explosives on the basis of minor plasticizer composition. These results will complement those obtained when using established methods for explosive characterization.

2. MATERIALS AND METHODS

2.1 Materials

The chemical structures of the plasticizers included in this study are shown in Figure 1. Three plasticizers are present in Semtex: tributyl citrate (*p1*), dioctyl phthalate (*p5*), and phthalate (*p4*). Two plasticizers are present in C-4 plastic explosive mixtures: dioctyl sebacate (*p2)* and bis(2-ethylhexyl) adipate (*p3*). The plasticizers and the serum albumin proteins (BSA and human serum albumin [HSA]) were obtained from Sigma-Aldrich Co. (St. Louis, MO). The NMR solvents used were 99.95% deuterium oxide (D_2O) and dimethyl-d_6 sulfoxide (DMSO-d_6), which were from Cambridge Isotope Laboratories (Tewksbury, MA), and ethanol-d_6, which was from Sigma-Aldrich.

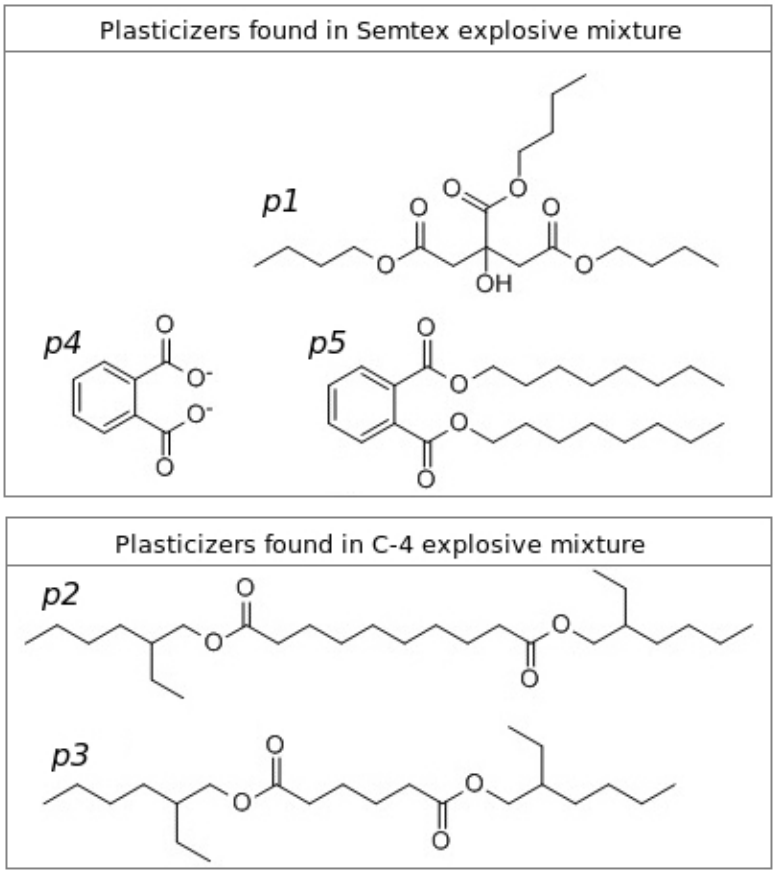

Figure 1. Structures of plasticizers: tributyl citrate (*p1*), phthalate (*p4*), dioctyl phthalate (*p5*), dioctyl sebacate (*p2*), and bis(2-ethylhexyl) adipate (*p3*).

2.2 Sample Preparation

Initially, NMR samples containing only serum albumin protein in 480 µL of phosphate buffer (20 mM sodium phosphate, pH 7.0, with 50 mM sodium chloride) were lyophilized three times and redissolved in 480 µL of D_2O. Subsequently, 120 µL of deuterated co-solvent (ethanol-d_6 or DMSO-d_6) was added to the NMR tube to a final volume of 600 µL. The amount of co-solvent was optimized to dissolve the water-insoluble plasticizers while the protein remained folded in the solution. Finally, plasticizers were added to the samples in aliquots of 5–10 µL each to avoid dilution effects from concentrated stock solutions prepared in the corresponding co-solvent. STD NMR data for the plasticizer were acquired in the presence of either BSA or HSA protein at concentrations of 0.1 to 1.0 µM. Although BSA and HSA exhibit significant (24%) differences in amino acid sequence,[10] in initial tests, we observed almost identical STD effects for the plasticizers in the presence of either protein. The final results presented in this study were obtained in the presence of BSA protein. Experimental conditions were further tested by collecting STD data for samples containing either ethanol-d_6 or DMSO as a co-solvent, at 15 and 25 °C, respectively. Slightly better STD enhancements were observed in the presence of ethanol-d_6 at 15 °C. The data were collected at the optimized protein-to-plasticizer molar ratio of 1:1000.

The reference sample, which contained soil, was prepared as follows: 0.5 g of soil was extracted with a 50:50 water–ethanol mixture. The sample was vortexed, filtered, and lyophilized three times. It was then redissolved in the NMR sample, which contained all five plasticizers (each at a 1.0 mM concentration), and the BSA protein (at a 1.0 µM concentration) in 600 µL of NMR buffer, which contained 20% ethanol-d_6.

The samples containing unknown explosive mixtures were prepared as follows: 0.5 g of each sample was extracted with a 50:50 water–ethanol mixture. The sample was vortexed, filtered, and lyophilized three times. It was then redissolved in 600 µL of NMR buffer, which contained 20% ethanol-d_6. Finally, BSA protein (at a 1.0 µM final concentration in 600 µL total volume) was added to the NMR sample in 2 µL aliquots to avoid sample dilution.

2.3 NMR Experiments

All NMR spectra were obtained on a Bruker AVHD 500 MHz spectrometer (Bruker Corp.; Billerica, MA) equipped with a 5 mm CPQNP Z-gradient CryoProbe at 25 or 15 °C.

One-dimensional proton (1D ^{1}H) spectra for each plasticizer, at a 2 mM concentration in DMSO-d_6, were obtained at 25 °C with 32,768 data points, 128 scans, a relaxation delay of 3.0 s, and a receive gain of 128.

The STD NMR experiments were performed as described by Mayer and Meyer.[9] Selective protein saturation, on-resonance at 0.0 ppm and off-resonance at 30 ppm, was achieved by a train of 40 Gaussian pulses of 50 ms each separated by 1 ms delays. Irradiation power corresponded to an 86 Hz field strength, for a total saturation time of 2.04 s. The Watergate (Water suppression through GrAdient Tailored Excitation) 3–9–19 pulse sequence was applied to suppress the residual water signal. The data were collected as a pseudo-2D NMR experiment in an interleaving manner to compensate for temperature and instrument instability effects, with 32,768 data points, 1024 scans, a 2.0 s relaxation delay, a 6510 Hz sweep width, and a receiver gain of 128. The on- and off-resonance free induction decays were stored and processed separately. The STD spectra were obtained by subtraction of the off- and on-resonance spectra. All NMR data were processed and analyzed using TopSpin 3.2 software (Bruker).

3. RESULTS AND DISCUSSION

To establish the feasibility of STD NMR as a tool for detection and identification of Semtex and C-4 plastic explosive mixtures on the basis of plasticizer composition, we first performed 1D ^{1}H and 1D STD NMR experiments of control samples. The control samples included the three plasticizers found in Semtex (*p5*, *p4*, and *p1)* and the two plasticizers found in C-4 (*p2* and *p3*), alone and in different combinations. To complete the suite of control tests, which was necessary to fully characterize the behavior of our reference system, we performed analog experiments using the same control samples but in the presence of soil. We demonstrated the validity of our novel approach by analyzing two unknown explosive samples that were sent to our laboratory for evaluation and characterization.

3.1 1D ^{1}H and 1D STD NMR of Control Samples

Because the ultimate goal of this study was to unambiguously detect a given plasticizer even when all plasticizers were present in a solution, it was crucial that there be at least one characteristic signature resonance for each plasticizer in a free-of-overlap spectral region. The observed signature resonances for *p1, p2, p3, p4,* and *p5* can be used as targets for detection and discrimination of the Semtex and C-4 plastic explosive mixtures in unknown analytes.

Figure 2 shows the 1D ^{1}H NMR spectra for individual plasticizers found in Semtex (*p5*, *p4,* and *p1*; blue traces) and C-4 (*p2* and *p3*; green traces) as well as the spectrum for all plasticizers together in the NMR sample (red trace). The boxed spectral regions A, B, and C at the top of the figure indicate the signature resonances for each plasticizer and are expanded for clarity. These data demonstrate that in an unknown analyte, the presence of Semtex can be successfully targeted by the signature peaks for *p5* and *p4* in the 7.5–8.0 ppm range (A), the *p5* triplet at 4.24 ppm (B), and the two *p1* doublets in the 2.7–3.0 ppm range (C). The C-4 plastic explosive mixture can be identified by the presence of characteristic triplets for *p2* and *p3* in the 2.2–2.5 ppm range (C) together with the isolated doublet for *p3* at 4.02 ppm (B). Additionally, these results clearly demonstrate that adequate discrimination of Semtex and C-4 plastic explosive mixtures was possible when both were present in the sample because the plasticizers *p5*, *p4,* and *p1* have characteristic spectral regions that are distinct from those observed for the plasticizers *p2* and *p3*.

Thus, the results from the 1D ^{1}H spectra for the control sample presented in Figure 2 demonstrated reliable identification of each plasticizer. This identification enabled the unambiguous discrimination of Semtex and C-4 plastic explosive mixtures, even when both were present in a sample that was free of interference signals in the signature spectral regions of the plasticizers.

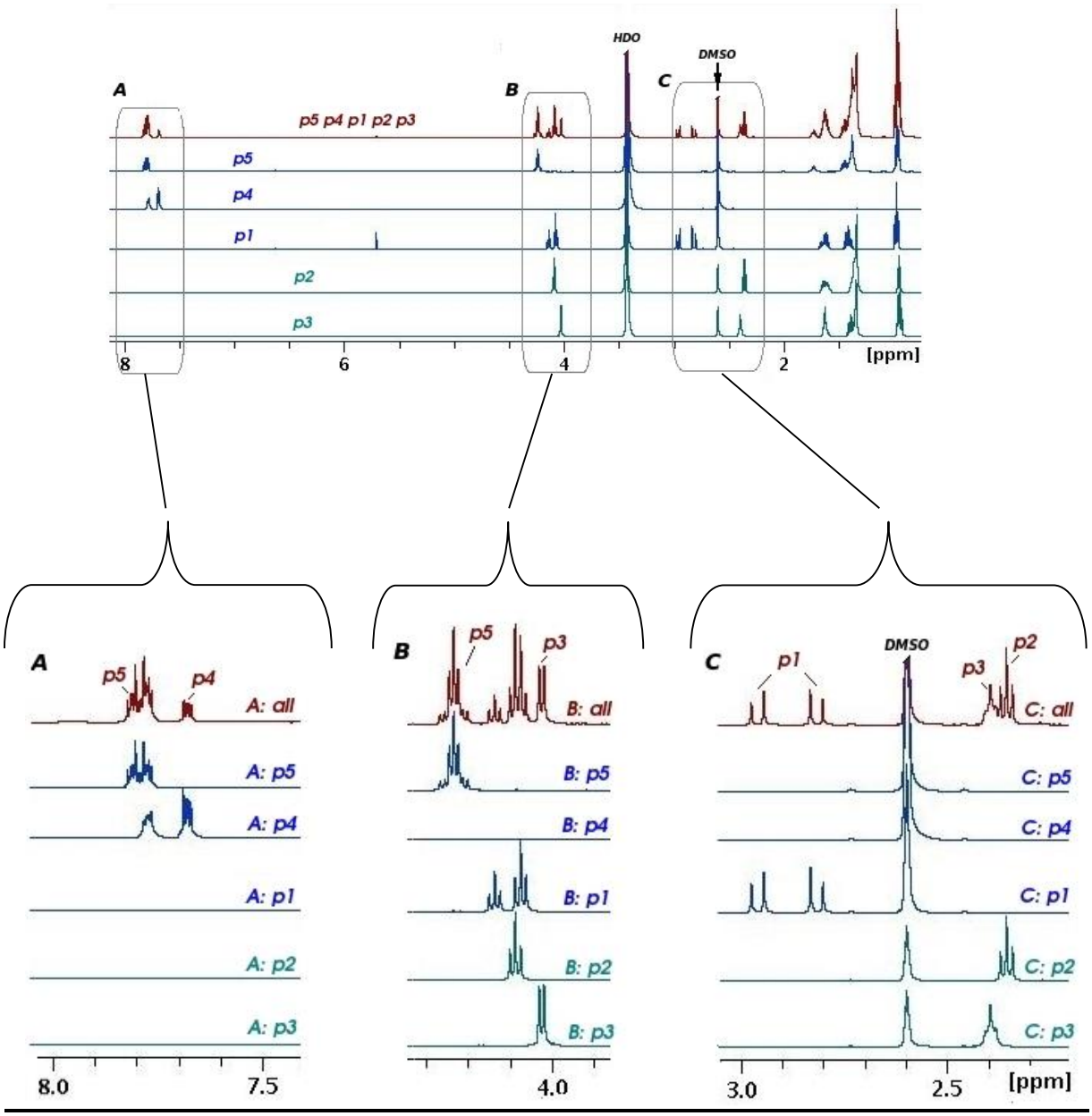

Figure 2. 1D ^{1}H NMR spectrum in DMSO-d_6 for each plasticizer found in Semtex (*p5*, *p4*, and *p1*; blue) or C-4 (*p2* and *p3*; green) plastic explosive mixtures. Top trace (red) represents the 1D ^{1}H NMR for all plasticizers present together in the NMR sample (1.0 mM concentration for each). Boxed spectral regions (A, B, and C) indicate the free-of-overlap spectral range for each plasticizer, which can be used for plasticizer identification when all are present in an unknown mixture.

Having defined the signature resonances for each plasticizer in the 1D ^{1}H spectra for detection and identification purposes, we demonstrated that 1D STD NMR retains the information of the 1D ^{1}H spectra and enables effective discrimination of the analyte signals in overcrowded 1D ^{1}H spectra of multicomponent samples. In the receptor-ligand system studied herein, the spectral editing was a result of the specific binding of the plasticizers to BSA protein. Under the conditions of the STD experiment, magnetization was transferred from the BSA receptor to the bound plasticizers, and STD signals were observed exclusively for the protons of the plasticizers at the binding interface. Because all proton signals from contaminations or other components of the explosive mixture did not exhibit STD effects, their NMR signals were effectively cancelled.

We compared the 1D ^{1}H and 1D STD NMR spectra for pure samples containing only BSA receptor and a given plasticizer at a 1:1000 molar ratio (Figure 3, top). Partial expansions of the signature frequency regions (A, B, C, and D) are shown for clarity. The data illustrate that the 1D STD spectrum exhibited the 1D ^{1}H spectrum chemical shift information and therefore could be successfully used as signature resonances for plasticizer identification. However, as shown in Figure 3, the relative intensities of the 1D STD signals were unique as compared with those for the proton NMR (1D ^{1}H); this was most pronounced for the *p2* resonances shown in Figure 3, B and D. In general, because of the differing nature of the interactions under the experimental conditions, it would be expected that the STD signals would exhibit resonance intensities different from those of the corresponding signals in the 1D ^{1}H data. In the 1D ^{1}H spectrum, resonance intensity depended on the number of equivalent protons in the molecule. In the 1D STD enhancements, relative intensity was determined by distance-dependent, protein-to-ligand through-space interactions. Thus, stronger or weaker STD effects were observed for ligand protons closer or further away from the protein, respectively. Despite the intensity change for the *p2* resonances, the characteristic pattern of three triplets was preserved in their 1D STD signals. This was distinct from the *p3* signature resonance, as illustrated in the expanded spectral regions of Figure 3, B and C.

In summary, as analogs to the 1D ^{1}H signature resonances, the signature STD effects of the plasticizers can be effectively used as reliable targets for their identification and discrimination.

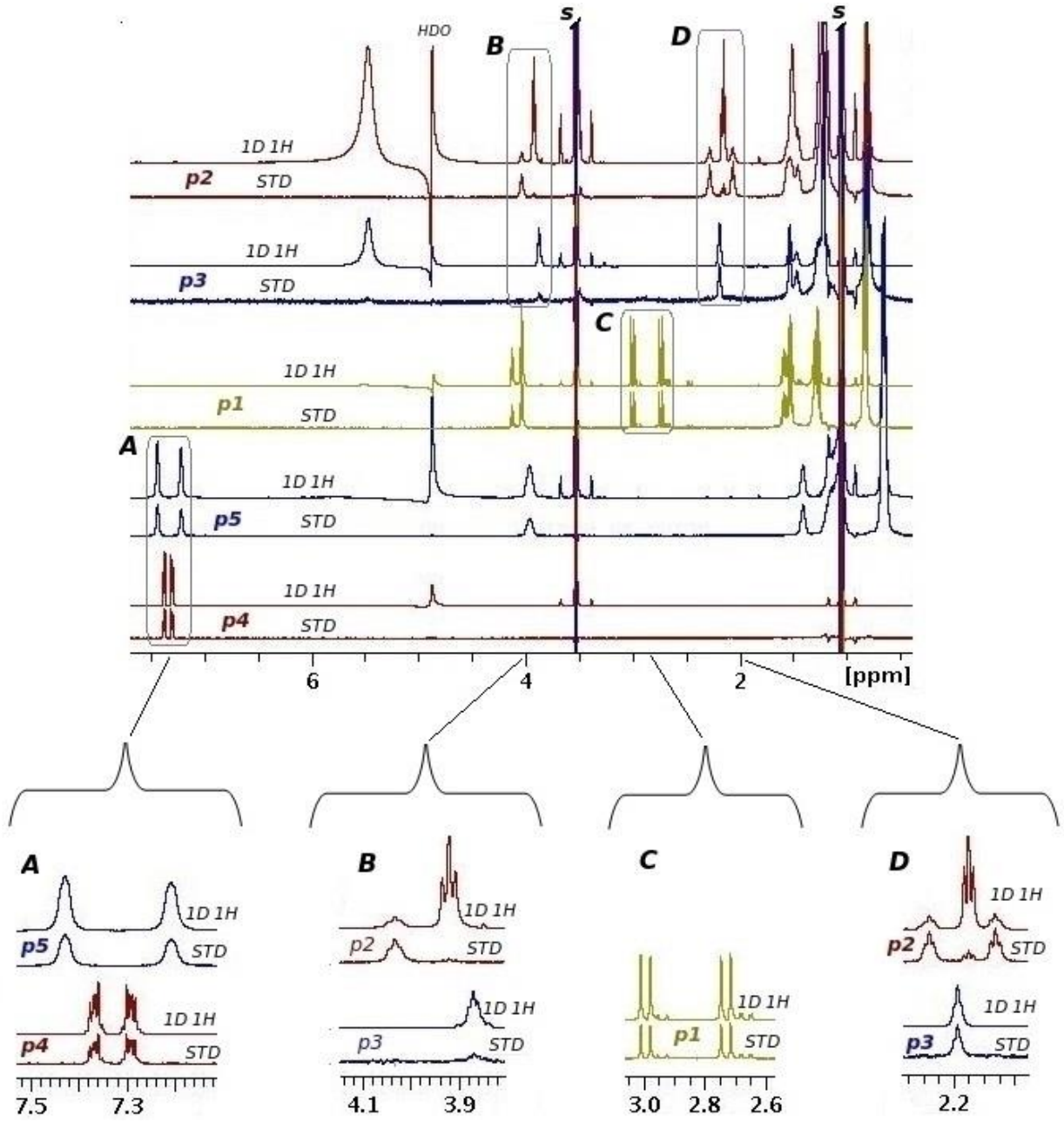

Figure 3. Comparison of 1D ^{1}H and 1D STD NMR spectra for each separate plasticizer (1.0 mM concentration) in the presence of 1 µM BSA protein. All spectra were collected in 80% D_2O buffer (50 mM phosphate buffer, pH 7) with 20% ethanol-d_6 as a co-solvent (indicated by *s*). Boxed spectral regions (A, B, C, and D) are expanded for clarity. The 1D ^{1}H and 1D STD NMR spectra were almost identical for each plasticizer except for *p2*, which showed significant change in the relative intensities of the STD resonances as compared with the corresponding 1D proton resonances (showed in the expanded ranges [B and D]). See text for explanation.

To assess the effect of the presence of all plasticizers in solution on the STD signature signals, STD spectra of reference materials were obtained by consecutive addition of small aliquots of each plasticizer in the NMR sample. Figure 4A shows the 1D STD spectra obtained from samples containing different combinations of *p5*, *p4*, and *p1* (A: a, b, and c), or *p2* and *p3* (A: d, e, and f), and the 1D STD spectra for all of them together (A: g). For reference, the corresponding signature resonances are indicated in the top spectrum.

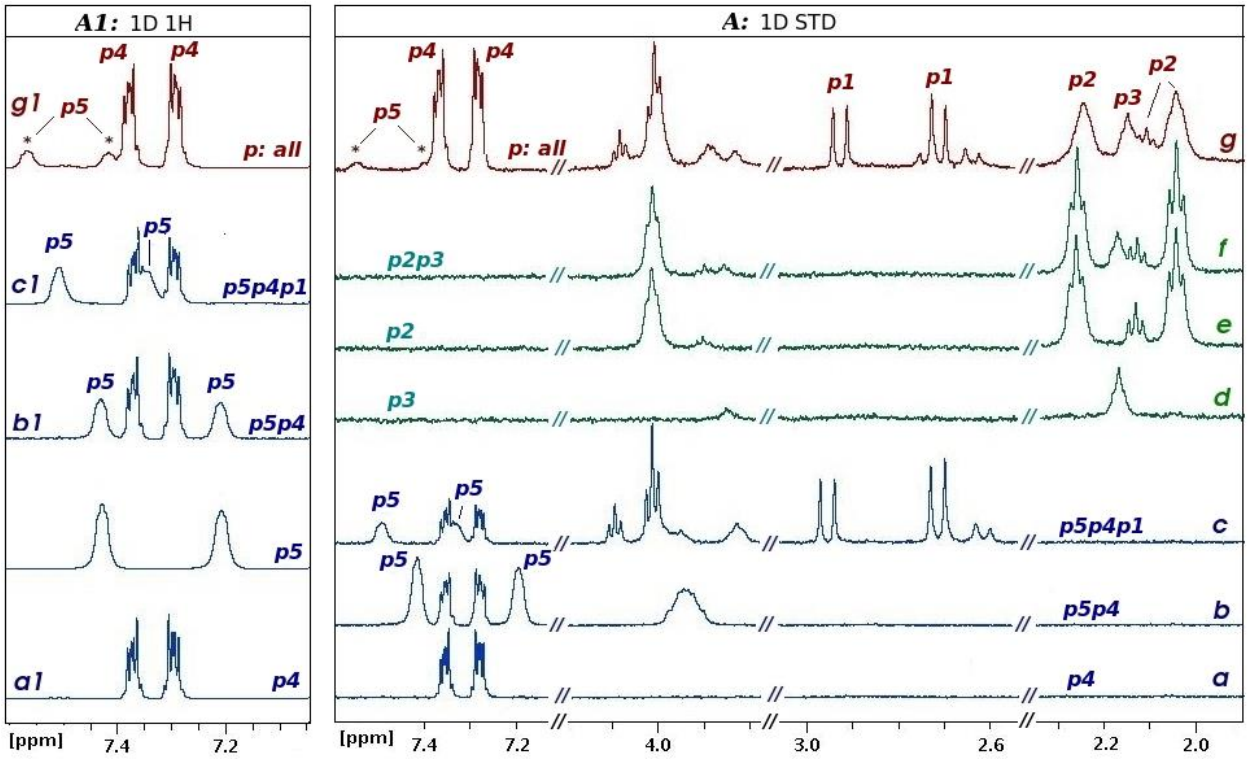

Figure 4. Effects of the presence of all plasticizers in solution on the STD signature signals for a given plasticizer. (A) 1D STD spectra for plasticizers *p1*, *p2*, *p3*, *p4*, and *p5* in the NMR samples, each alone or in different combinations with the others. In all cases, the characteristic shifts and line patterns necessary for the identification of each plasticizer in the mixture were preserved. Signature resonances are labeled in the top spectrum (red). Frequency shifts observed for the *p5* doublet in the 7.1–7.6 ppm region were attributed to presumed aggregation of *p5* (as evidenced by the broad line widths) because of its limited solubility in the applied NMR buffer and would not preclude identification of the *p5* plasticizer. (A1) The portion of the 1D ^{1}H spectra that contains the characteristic NMR frequencies (7.1–7.6 ppm) for *p4* and *p5* plasticizers. The same shift patterns are exhibited in the 1D STD spectra (A).

The 1D STD spectra shown in Figure 4A revealed no perturbation of the STD effects for *p1*, *p2*, *p3*, and *p4* upon consecutive addition of plasticizers, as was evidenced by the identical characteristic chemical shifts and line shapes of the signature resonances. However, as a result of consecutive addition of plasticizers, downfield chemical shifts of the characteristic *p5* resonances in 7.0–7.5 ppm range were observed in both the 1D STD spectra (Figure 4A: c and g)

and the 1D ^{1}H spectra. These are shown for comparison (Figure 4A1: c1 and g1). Another important feature is the much broader line widths of the *p5* resonances that were observed in the 7.0–7.5 ppm spectral region.

In general, broader NMR spectral lines are associated with either slower molecular tumbling or conformational exchange processes. In this study, we attributed the observed broader lines and the downfield chemical shifts for *p5* resonances to partial aggregation and molecular association, which result in slower molecular tumbling as a result of the limited solubility of *p5* in the applied NMR buffer. Because the line widths for *p5* and *p4* were preserved under the experimental conditions, the different line shapes can be used as additional signatures to aid in their discrimination in the 7.0–7.7 ppm range.

These results clearly demonstrate that the signature STD effects of the plasticizers, together with their characteristic line shapes, can be used as reliable indicators for the presence of Semtex and C-4 explosives in complex analytes. Equally important is that the distinct signature resonances for plasticizers *p1, p2, p3, p4,* and *p5* provide a straightforward means for discrimination of Semtex and C-4 plastic explosive mixtures when both are present in an unknown analyte.

3.2 1D ^{1}H and 1D STD NMR of Control Samples in the Presence of Soil

Having established the utility of the STD NMR approach for control samples containing plasticizers and BSA protein only, we investigated its validity for authentic samples that were potentially contaminated with soil and other typical constituents of the explosive mixtures of interest. STD data were collected as described (Section 2.3) but in the presence of soil. Samples were prepared as previously described (Section 2.2). Results are illustrated in Figure 5A. A comparison of the 1D ^{1}H spectra for reference samples that contained all five plasticizers in the presence and absence of soil (Figure 5A: a and b, respectively) revealed strong proton signal interference in the 3.2–4.5 and 1.7–2.5 ppm spectral regions due to the presence of soil. In contrast, the corresponding STD spectra obtained in the presence and absence of soil (Figure 5A: c and d, respectively) were nearly identical. This demonstrated the ability of the STD methodology to completely eliminate strong signals due to interference. This allows unequivocal identification of each plasticizer for identification and discrimination of Semtex and C-4 explosives in unknown samples. An expansion (plotted at a higher vertical scale) of the signature spectral regions of plasticizers *p5* and *p4* is shown in Figure 5A1.

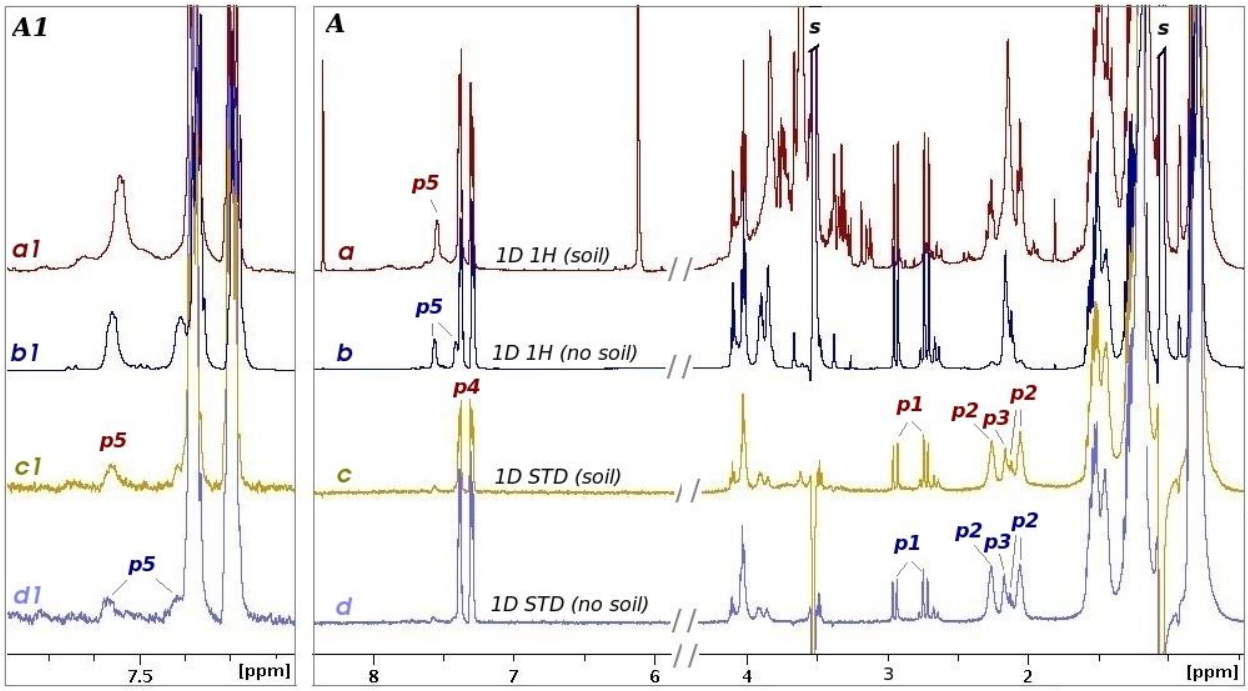

Figure 5. Effects of the presence of soil in control samples on the STD signature resonances for plasticizers *p1*, *p2*, *p3*, *p4*, and *p5*. (A) Comparison of the NMR spectra for the reference sample, which contained all five plasticizers in the presence of soil (a: 1D^{1}H; and c: 1D STD), and the spectra for the same sample with no soil (b: 1D^{1}H; and d: 1D STD). The STD spectrum for plasticizers in the presence of soil (c) showed excellent cancellation of all strong background signals and exhibited only proton resonances for the plasticizers, which were identical to the corresponding resonances in the reference STD spectrum (d). (A1) Expansion of the spectral range in the 7.2–7.5 ppm region of (A).

3.3 1D ^{1}H and 1D STD NMR of Samples Containing Unknown Plastic Explosive Mixtures

Finally, our novel approach was employed for the analysis of two authentic explosives samples sent to our laboratory for full characterization. The NMR samples were prepared as described (Section 2.2).

Shown in Figure 6 are the 1D ^{1}H and 1D STD NMR spectra for unknown samples UN-1 and UN-2 (in red) and the reference 1D STD spectra for *p2*, *p3*, and *p2* and *p3* (in teal), with expansions of the outlined diagnostic frequency regions A and B. The 1D ^{1}H spectra for these particular unknowns showed almost no interference proton peaks except for the one at 6.2 ppm. In this favorable case, the 1D ^{1}H and the 1D STD NMR spectra provided identical information. Comparison of the 1D STD spectra for the unknown samples with the STD spectra for the control *p2* and *p3* samples clearly indicates the presence of plasticizer *p2* only in sample UN-1 and the presence of both *p2* and *p3* in sample UN-2.

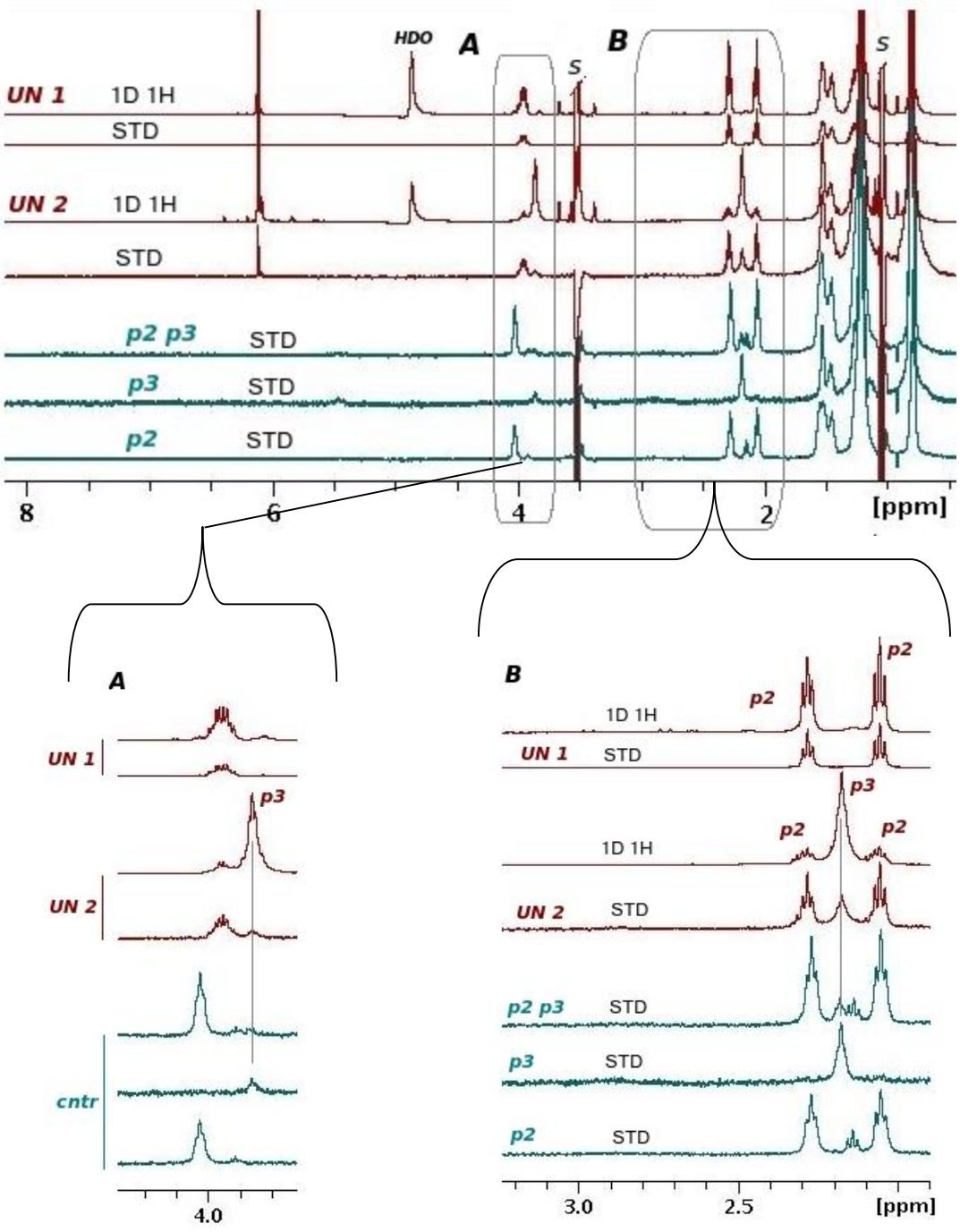

Figure 6. 1D ^{1}H and 1D STD NMR spectra of two unknown plastic explosive mixtures, UN-1 and UN-2 (red) and control 1D STD spectra (green). Boxed regions are expanded for clarity. Comparison with the control STD spectra confirmed the presence of *p2* plasticizer only in explosive mixture UN-1, whereas sample UN-2 contained both *p2* and *p3*, which is consistent with the C-4 plastic explosive mixture.

These results are consistent with the presence of C-4 plastic explosive mixture in the UN-2 sample. However, additional information based on other analytical methods would be required to eliminate the possibility that C-4 plastic explosive mixture was in the UN-1 sample. The Semtex explosive mixture was not present in either of the two unknown samples.

4. CONCLUSION

We have developed a novel method for the detection and differentiation of Semtex and C-4 plastic explosive mixtures by identification of their minor plasticizer composition on the basis of chemical shift information (signature resonances) as derived by 1D STD NMR. NMR chemical shifts are an excellent diagnostic tool because they reliably reflect the overall or local structural features of a molecule under many experimental and sample conditions. The 1D STD NMR approach presented herein retained the valuable information derived by use of 1D ^{1}H NMR and at the same time simplified the overcrowded 1D ^{1}H spectra of multi-component systems, where it often becomes a challenging task to correctly identify an analyte of interest. In this study, we demonstrated that the use of 1D STD NMR enables selective diagnosis for plasticizers in Semtex and C-4 plastic explosive mixtures on the basis of their specific binding to BSA protein. The method provided direct, unambiguous structural identification of the plasticizers on the atomic level and enabled straightforward identification and discrimination of the corresponding Semtex and C-4 plastic explosive mixtures in authentic samples. The 1D STD NMR methodology is a valuable addition to the already-established methods for detection and attribution of plastic explosive mixtures.

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ACRONYMS AND ABBREVIATIONS

1D 1H	one-dimensional proton
1D STD	one-dimensional saturation transfer difference
AN	ammonium nitrate
BSA	bovine serum albumin
EGDN	ethylene glycol dinitrate
HDO	partially deuterated water
HMX	1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane
HSA	human serum albumin
NC	nitrocellulose
NMR	nuclear magnetic resonance
p1	tributyl citrate
p2	dioctyl sebacate
p3	bis(2-ethylhexyl) adipate
p4	phthalate
p5	dioctyl phthalate
PETN	pentaerythritol tetranitrate
RDX	1,3,5-trinitro-1,3,5-triazacyclohexane
STD	saturation transfer difference
TNT	2,4,6-trinitrotoluene

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